

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

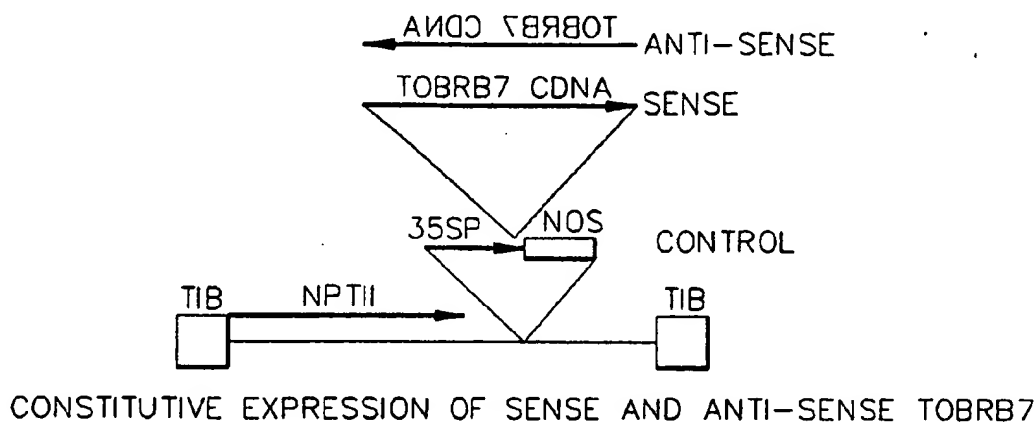
**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>5</sup> : C12N 15/82, A01H 5/00, A01N 65/00		A1	(11) International Publication Number: WO 94/17194
			(43) International Publication Date: 4 August 1994 (04.08.94)
(21) International Application Number: PCT/US94/00217		(74) Agents: SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).	
(22) International Filing Date: 6 January 1994 (06.01.94)			
(30) Priority Data: 007,998 21 January 1993 (21.01.93) US		(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(60) Parent Application or Grant (63) Related by Continuation US 007,998 (CON) Filed on 21 January 1993 (21.01.93)		Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.	
(71) Applicant (for all designated States except US): NORTH CAROLINA STATE UNIVERSITY [US/US]; 103 Holladay Hall, Campus Box 7003, Raleigh, NC 27695-7003 (US).			
(72) Inventors; and (75) Inventors/Applicants (for US only): CONKLING, Mark, A. [US/US]; 5313 April Wind Drive, Fuquay-Varina, NC 27526 (US). OPPERMAN, Charles, H. [US/US]; 500 St. Andrews Court, Raleigh, NC 27615 (US). ACEDO, Gregoria, N. [US/US]; 22 Westridge Drive, Durham, NC 27713 (US). SONG, Wen [US/US]; 2702 Vanderbilt Avenue, Raleigh, NC 27607 (US).			

(54) Title: NEMATODE-RESISTANT TRANSGENIC PLANTS



CONSTITUTIVE EXPRESSION OF SENSE AND ANTI-SENSE TOBRB7

## (57) Abstract

Nematode-resistant transgenic plants are disclosed. The plants comprise plant cells containing a DNA construct comprising a transcription cassette, which construct comprises, in the 5' to 3' direction, a promoter operable in the plant cells, and a DNA comprising at least a portion of a DNA sequence encoding a nematode-inducible transmembrane pore protein in either the sense or antisense orientation. Intermediates for producing the same along with methods of making and using the same are also disclosed. In an alternate embodiment of the invention, the sense or antisense DNA is replaced with a DNA encoding an enzymatic RNA molecule directed against the mRNA transcript of a DNA sequence encoding a nematode-inducible transmembrane pore protein.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

## NEMATODE-RESISTANT TRANSGENIC PLANTS

This invention was made with government support under grant number DMB 88-11077 from the National Science Foundation. The Government may have certain rights to this invention.

5

### Field of the Invention

This invention relates to methods of controlling plant-parasitic nematodes by application of recombinant DNA technology and the production of transgenic plants.

### Background of the Invention

10

World-wide, plant-parasitic nematodes are among the most devastating pathogens of life sustaining crops. In 1984, nematodes accounted for more than fifty billion dollars (US) in economic losses. The United States' portion of this figure alone is almost six billion dollars.

15

Genetic resistance to certain nematode species is available in some cultivars, but these are restricted in number, and the availability of cultivars with both desirable agronomic features and resistance is limited. . In addition,

20

traditional methods for plant breeding require 5-10 years to produce a viable cultivar, while the need for new nematode control tools is immediate and critical.

-2-

The major means of nematode control has been the application of chemical nematicides. During 1982, in the United States alone over 100 million pounds of nematicide were applied to crops. Chemical nematicides are generally highly toxic compounds known to cause substantial environmental impact. In the past several years, issues such as ground water contamination, mammalian and avian toxicity, and residues in food have caused much tighter restrictions on the use of chemical nematicides. Unfortunately, in many situations there is no alternative available for growers who rely upon nematicides to protect their crop from root-knot and cyst nematodes. Accordingly, there is a continuing need for new ways to combat nematodes in plants.

15

#### Summary of the Invention

A first aspect of the present invention is a DNA construct comprising a transcription cassette. The construct comprises, in the 5' to 3' direction, (a) a promoter operable in a plant cell, (b) a DNA comprising at least 15 nucleotides of a DNA sequence encoding a nematode-inducible transmembrane pore protein in either the opposite orientation for expression (i.e., an antisense DNA) or the proper orientation for expression (i.e., a sense DNA), and (c) optionally, but preferably, a termination signal. The promoter may be one which is constitutively active in plant cells, selectively active in plant root tissue cells, or a nematode-responsive element such as the nematode-responsive element of the Tobacco RB7 (TobRB7) promoter. Such constructs may be carried by a plant transformation vector such as an *Agrobacterium tumefaciens* vector, which are in turn used to produce recombinant plants.

A second aspect of the present invention is, accordingly, a nematode-resistant transgenic plant. The plant comprises cells containing a DNA construct comprising a transcription cassette as described above.

In particular embodiments of the invention, DNA encoding a nematode-inducible transmembrane pore protein may be selected from the group consisting of: (a) isolated DNA having the sequence given herein as SEQ ID NO:1 (which  
5 DNA encodes the nematode-inducible transmembrane pore protein given herein as SEQ ID NO:2) or SEQ ID NO:6 (which is a genomic DNA encoding the nematode-inducible transmembrane pore protein given herein as SEQ ID NO:7, which is the same as SEQ ID NO:2); (b) isolated DNA which  
10 hybridizes to isolated DNA of (a) above and which encodes a nematode inducible transmembrane pore protein (which isolated DNA is preferably at least 50% homologous with an isolated DNA of (a) above; and which pore protein is preferably at least 60% homologous with a pore protein of  
15 (a) above); and (c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encode a nematode-inducible transmembrane pore protein. A specific example of such a DNA, in antisense configuration  
20 for carrying out the present invention, is given herein as SEQ ID NO:3.

Additionally, in particular embodiments of the invention, DNA encoding a nematode-responsive element may be selected from the group consisting of: (i) isolated DNA  
25 having the sequence given herein as SEQ ID NO:5; and (ii) isolated DNA which hybridizes to isolated DNA of (i) above and which encodes a nematode responsive element (which is preferably at least 60% homologous to isolated DNA of (i) above; and which are preferably at least 10 or 15  
30 nucleotides in length) (this definition is intended to include fragments of (i) above which retain activity as nematode-responsive elements).

The foregoing and other objects and aspects of this invention are explained in detail in the drawings  
35 herein and the specification set forth below.

-4-

Brief Description of the Drawings

Figure 1 illustrates a pair of DNA constructs comprising transcription cassettes, one in which the TobRB7 cDNA in sense configuration under the transcriptional control of a CaMV 35S promoter, and the other with a TobRB7 cDNA in antisense configuration under the transcriptional control of a CaMV 35S promoter. A nos 3' termination sequence and a neomycin phosphotransferase II (NPT-II) selectable marker for imparting kanamycin resistance is provided in both cases. The border regions of the Ti plasmid into which the cassette is inserted are indicated as "TiB".

Figure 2 illustrates transcription cassettes much like those illustrated in Figure 1 above, except that the constitutively active CaMV35S promoter is replaced with either the element TobRB7 Δ0.6 which is selectively active in root tissue cells or the nematode-responsive element TobRB7 Δ0.3.

Detailed Description of the Invention

The present invention is employed to combat nematodes, particularly the root knot nematodes (*Meloidogyne* spp.) and the cyst nematodes (*Globodera* spp. and *Heterodera* spp.). These nematodes have similar life cycles. Root-knot nematodes are sedentary endoparasites with an extremely intimate and complex relationship to the host plant. The infective second stage juvenile (J2) is free in the soil. Upon location of a host root, the J2 penetrates the root intercellularly in the region just posterior to the root cap and migrates to the developing vascular cylinder. The nematode then orients itself parallel to the cylinder and injects glandular secretions into the plant cells surrounding its head, resulting in the initiation of nematode feeding cells. These 5-7 cells undergo rapid nuclear divisions, increase tremendously in size, and become filled with pores and cell wall invaginations. The feeding site cells, or "giant cells",

-5-

function as super transfer cells to provide nourishment to the developing nematode. During this time, the nematode loses the ability to move and swells from the normal eel shaped J2 to a large, pear shaped adult female. As the  
5 nematode feeds on the giant cells, parthenogenic reproduction results in the the disposition of 300-1000 eggs. This entire process occurs over the span of 20-30 days, and root-knot nematodes may complete as many as 7 generations during a cropping season. The life cycle of  
10 the cyst nematode is essentially the same, except that its feeding site is referred to as a "syncytia", and it undergoes sexual reproduction.

Nematode-inducible transmembrane pore proteins are pore proteins the expression of which is increased in  
15 cells upon infection of a plant containing the cells by a plant-parasitic nematode at a position adjacent those cells. Increased expression of such pore proteins is required by the nematode in establishing a feeding site capable of passing nutrients from the plant to the  
20 nematode. In general, and as explained in greater detail below, DNA encoding nematode-inducible transmembrane pore proteins include DNA which is 50% homologous or more with DNA having the sequence given herein as SEQ ID NO:1 or SEQ ID NO:6. With respect to the protein, DNA encoding  
25 nematode-inducible transmembrane pore proteins encode a protein which, in amino acid content, is about 60% homologous or more, or preferably about 70% homologous or more, with the protein having the amino acid sequence given herein as SEQ ID NO:2. Determinations of homology are made  
30 with the two sequences (nucleic acid or amino acid) aligned for maximum matching. Gaps in either of the two sequences being matched are allowed in maximizing matching. Gaps lengths of 10 or less are preferred, gap lengths of 5 or less are more preferred, and gap lengths of 2 or less still  
35 more preferred.

Differential hybridization procedures are available which allow for the isolation of cDNA clones



-6-

whose mRNA levels are as low as about 0.05% of poly(A<sup>+</sup>)RNA. See M. Conkling et al., *Plant Physiol.* 93, 1203-1211 (1990). In brief, cDNA libraries are screened using single-stranded cDNA probes of reverse transcribed mRNA from plant tissue (i.e., roots and leaves). For differential screening, a nitrocellulose or nylon membrane is soaked in 5xSSC, placed in a 96 well suction manifold, 150  $\mu$ L of stationary overnight culture transferred from a master plate to each well, and vacuum applied until all liquid has passed through the filter. 150  $\mu$ L of denaturing solution (0.5M NaOH, 1.5 M NaCl) is placed in each well using a multiple pipetter and allowed to sit about 3 minutes. Suction is applied as above and the filter removed and neutralized in 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl. It is then baked 2 hours in vacuo and incubated with the relevant probes. By using nylon membrane filters and keeping master plates stored at -70°C in 7% DMSO, filters may be screened multiple times with multiple probes and appropriate clones recovered after several years of storage.

For example, to isolate genes whose expression is induced or enhanced by nematode infection, a cDNA library of mRNA isolated from nematode infected tobacco roots is constructed. The roots are staged such that mRNA is isolated at the time of giant cell initiation. The library is then screened by the procedures given above using single stranded cDNA probes of mRNA isolated from nematode-infected and control roots. Those cDNA clones exhibiting differential expression are then used as probes on tobacco genomic Southern blots (to confirm the cDNA corresponds to tobacco and not nematode transcripts) and Northern blots of root RNA from infected and control tissue (to confirm differential expression). Those clones exhibiting differential expression are then used as probes to screen an existing tobacco genomic library. Essentially the same procedure is carried out with plants other than tobacco and nematodes (or other pathogens) other than root-

-7-

knot nematodes. The procedure is useful for identifying promoters induced by cyst nematodes, in which case the roots are staged such that mRNA is isolated at the time of syncytia initiation. For example, a potato-cyst nematode (5 *Globodera* spp.) inducible promoter is isolated from potato plants (*Solanum tuberosum*) in accordance with the foregoing procedures.

We have probed a wide variety of dicotyledonous and monocotyledonous plants at low stringency with TobRB7 probes and have found that most (if not all) plants contain 10 a TobRB7 analog. We have already identified by low stringency hybridization such a root-specific cDNA analog from *Arabidopsis thaliana* (AtRB7) (Yamamoto, Cheng, and Conkling 1990 *Nucl. Acids Res.* 18: 7449).

15 Nematode-inducible transmembrane pore proteins employed in carrying out the present invention include proteins homologous to, and having essentially the same biological properties as, the nematode-inducible pore protein Tobacco RB7 disclosed herein as SEQ ID NO:2 (the 20 same as SEQ ID NO:7). This definition is intended to encompass natural allelic variations in the pore protein. Cloned genes employed in carrying out the present invention may code for a nematode-inducible pore protein of any species of origin, including tobacco, soybean, potato, 25 peanuts, pineapple, cotton, and vegetable crops, but preferably encode a nematode-inducible transmembrane pore protein of dicot origin. Thus, DNA sequences which hybridize to DNA of SEQ ID NO:1 or SEQ ID NO:6 and code on expression for a nematode-inducible transmembrane pore 30 protein may also be employed in carrying out the present invention. Conditions which will permit other DNA sequences which code on expression for a pore protein to hybridize to a DNA having the sequence given as SEQ ID NO:1 or SEQ ID NO:6 can be determined in a routine manner. For 35 example, hybridization of such sequences may be carried out under conditions of reduced stringency or even stringent conditions (e.g., conditions represented by a wash

-8-

stringency of 0.3 M NaCl, 0.03 M sodium citrate, 0.1% SDS at 60°C or even 70°C to DNA having the sequence given as SEQ ID NO:1 or SEQ ID NO:6 herein in a standard in situ hybridization assay. See J. Sambrook et al., Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory)). In general, such sequences will be at least 75% homologous, 80% homologous, 85% homologous, 90% homologous, or even 95% homologous or more with the sequence given herein as SEQ ID NO:1 or SEQ ID NO:6 (in the case of SEQ ID NO:6, which is a genomic sequence, such homology is with respect to the exons alone, though the homology may be considered with respect to both introns and exons). Determinations of homology are made with the two sequences aligned for maximum matching. Gaps in either of the two sequences being matched are allowed in maximizing matching. Gap lengths of 10 or less are preferred, gap lengths of 5 or less are more preferred, and gap lengths of 2 or less still more preferred.

Antisense DNAs in the present invention are used to produce the corresponding antisense RNAs. An antisense RNA is an RNA which is produced with the nucleotide bases in the reverse or opposite order for expression. Such antisense RNAs are well known. See, e.g., U.S. Patent No. 4,801,540 to Calgene Inc. In general, the antisense RNA will be at least 15 nucleotides in length, and more typically at least 50 nucleotides in length. The antisense RNA may include an intron-exon junction (i.e., one, two, or three nucleotides on either or both sides of the intron-exon junction). Antisense RNAs which include an intron-exon junction are constructed with reference to a genomic DNA sequence.

Sense DNAs employed in carrying out the present invention are of a length sufficient to, when expressed in a plant cell, suppress the native expression of a nematode-inducible transmembrane pore protein as described herein in that plant cell. Such sense DNAs may be essentially an entire genomic or complementary DNA encoding the nematode-

-9-

inducible transmembrane pore protein or a fragment thereof, with such fragments typically being at least 15 nucleotides in length.

5 In an alternate embodiment of the present invention, the sense or antisense DNA in the construct is replaced with a DNA encoding an enzymatic RNA molecule (i.e., a "ribozyme"), which enzymatic RNA molecule is directed against (i.e., cleaves) the mRNA transcript of a DNA encoding a nematode-inducible transmembrane pore  
10 protein as described hereinabove. DNA encoding enzymatic RNA molecules may be produced in accordance with known techniques. See, e.g., T. Cech et al., U.S. Patent No. 4,987,071 (the disclosure of which is to be incorporated herein by reference). Production of such an enzymatic RNA  
15 molecule and disruption of pore protein production combats the infection of plants by nematodes in essentially the same manner as production of an antisense RNA molecule: that is, by disrupting translation of mRNA in the cell which produces the pore protein.

20 Promoters employed in carrying out the present invention may be constitutively active promoters. Numerous constitutively active promoters which are operable in plants are available. A preferred example is the Cauliflower Mosaic Virus (CaMV) 35S promoter. In the  
25 alternative, the promoter may be a root-specific promoter or a nematode-responsive element, as explained in greater detail below.

Promoters which are selectively active in plant root tissue cells employed in carrying out the present  
30 invention include DNAs homologous to, and having essentially the same biological properties as, the Tobacco RB7 root-specific gene promoter disclosed herein as SEQ ID NO:4. This definition is intended to encompass natural  
35 allelic variations therein. Such elements may be of any species of origin, including tobacco, soybean, potato, peanuts, pineapple, cotton, and vegetable crops, but preferably are of dicot origin. Thus, DNA sequences which

-10-

hybridize to DNA of SEQ ID NO:4 and contain a root-specific gene promoter may also be employed in carrying out the present invention. Conditions which will permit other DNA sequences which code for a such an element to hybridize to a DNA having the sequence given as SEQ ID NO:4 can be determined in a routine manner. For example, hybridization of such sequences may be carried out under conditions as given above in connection with nematode-inducible transmembrane pore proteins. Such sequences will generally be at least 75% homologous, 80% homologous, 85% homologous, 90% homologous, or even 95% homologous or more with the sequence given herein as SEQ ID NO:4. Gaps may be introduced to maximize homology when determining homology, as discussed above. In addition, homology may be determined with respect to a 10 to 15 or even 25 or 50 base segment of a DNA having the sequence of SEQ ID NO:5 and capable of directing nematode-responsive transcription of a downstream DNA sequence (i.e., a structural gene or an antisense DNA) in a plant cell. By "base segment" is meant a continuous portion thereof which is of the indicated number of nucleotides in length.

Nematode-responsive elements employed in carrying out the present invention include DNAs homologous to, and having essentially the same biological properties as, the Tobacco RB7 nematode-responsive element disclosed herein as SEQ ID NO:5. This definition is intended to encompass natural allelic variations therein. Such elements may again be of any species of origin, including tobacco, soybean, potato, peanuts, pineapple, cotton, and vegetable crops, but preferably are of dicot origin. Thus, DNA sequences which hybridize to DNA of SEQ ID NO:5 and contain a nematode-responsive element may also be employed in carrying out the present invention. Conditions which will permit other DNA sequences which code for a such an element to hybridize to a DNA having the sequence given as SEQ ID NO:5 can again be determined in a routine manner. For example, hybridization of such sequences may be carried

-11-

out under conditions as given above in connection with nematode-inducible transmembrane pore proteins. Such sequences will generally be at least 75% homologous, 80% homologous, 85% homologous, 90% homologous, or even 95% homologous or more with the sequence given herein as SEQ ID NO:5. Gaps may be introduced to maximize homology when determining homology, as discussed above. In addition, homology may be determined with respect to a 10 to 15 or even 25 or 50 base segment of a DNA having the sequence of SEQ ID NO:5 and capable of directing nematode-responsive transcription of a downstream DNA sequence (i.e., a structural gene or an antisense DNA) in a plant cell.

DNA constructs, or "transcription cassettes," of the present invention include, 5' to 3' in the direction of transcription, a promoter as discussed above, a DNA operatively associated with the promoter, and, optionally, a termination sequence including stop signal for RNA polymerase and a polyadenylation signal for polyadenylase. All of these regulatory regions should be capable of operating in the cells of the tissue to be transformed. Any suitable termination signal may be employed in carrying out the present invention, examples thereof including, but not limited to, the nos terminator, the CaMV terminator, or native termination signals derived from the same gene as the transcriptional initiation region or derived from a different gene. The term "operatively associated," as used herein, refers to DNA sequences on a single DNA molecule which are associated so that the function of one is affected by the other. Thus, a promoter is operatively associated with a DNA when it is capable of affecting the transcription of that DNA (i.e., the DNA is under the transcriptional control of the promoter). The promoter is said to be "upstream" from the DNA, which is in turn said to be "downstream" from the promoter.

The transcription cassette may be provided in a DNA construct which also has at least one replication system. For convenience, it is common to have a

-12-

replication system functional in *Escherichia coli*, such as ColE1, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there will frequently be at least one marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host, while another marker may be employed for selection in a eukaryotic host, particularly the plant host. The markers may be protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; provide complementation, by imparting prototrophy to an auxotrophic host; or provide a visible phenotype through the production of a novel compound in the plant. Exemplary genes which may be employed include neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT), chloramphenicol acetyltransferase (CAT), nitrilase, and the gentamicin resistance gene. For plant host selection, non-limiting examples of suitable markers are NPTII, providing kanamycin resistance or G418 resistance, HPT, providing hygromycin resistance, and the mutated *aroA* gene, providing glyphosate resistance.

The various fragments comprising the various constructs, transcription cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system, and insertion of the particular construct or fragment into the available site. After ligation and cloning the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature and find particular exemplification in J. Sambrook et al.,

-13-

Molecular Cloning, A Laboratory Manual (2d Ed. 1989) (Cold Spring Harbor Laboratory).

5 Vectors which may be used to transform plant tissue with DNA constructs of the present invention include both *Agrobacterium* vectors and ballistic vectors, as well as vectors suitable for DNA-mediated transformation.

10 Methods of making recombinant nematode-resistant plants of the invention, in general, involve providing a plant cell capable of regeneration (the plant cell typically residing in a tissue capable of regeneration). The plant cell is then transformed with a DNA construct comprising a transcription cassette of the present invention (as described herein) and a recombinant nematode-resistant plant regenerated from the transformed plant cell. As explained below, the transforming step is carried out by bombarding the plant cell with microparticles carrying the transcription cassette, by infecting the cell with an *Agrobacterium tumefaciens* containing a Ti plasmid carrying the transcription cassette, or any other technique  
15 20 suitable for the production of a transgenic plant.

Numerous *Agrobacterium* vector systems useful in carrying out the present invention are known. For example, U.S. Patent No. 4,459,355 discloses a method for transforming susceptible plants, including dicots, with an  
25 *Agrobacterium* strain containing the Ti plasmid. The transformation of woody plants with an *Agrobacterium* vector is disclosed in U.S. Patent No. 4,795,855. Further, U.S. Patent No. 4,940,838 to Schilperoort et al. discloses a binary *Agrobacterium* vector (i.e., one in which the  
30 *Agrobacterium* contains one plasmid having the vir region of a Ti plasmid but no T region, and a second plasmid having a T region but no vir region) useful in carrying out the present invention.

35 Microparticles carrying a DNA construct of the present invention, which microparticle is suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present invention.



-14-

The microparticle is propelled into a plant cell to produce a transformed plant cell, and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practicing the present invention. Exemplary apparatus and procedures are disclosed in Sanford and Wolf, U.S. Patent No. 4,945,050, and in Christou et al., U.S. Patent No. 5,015,580. When using ballistic transformation procedures, the transcription cassette may be incorporated into a plasmid capable of replicating in or integrating into the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5  $\mu\text{m}$  gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art.

Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

-15-

Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the transcription cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques. A dominant selectable marker (such as *nptII*) can be associated with the transcription cassette to assist in breeding.

Some plants-parasitic nematodes from which plants may be protected by the present invention, and the corresponding plants which may be employed in practicing the present invention, are as follows: Alfalfa: *Ditylenchus dipsaci*, *Meloidogyne hapla*, *Meloidogyne incognita*, *Meloidogyne javanica*, *Pratylenchus* spp., *Paratylenchus* spp., and *Xiphinema* spp.; Banana: *Radopholus similis*, *Helicotylenchus multicinctus*, *Meloidogyne incognita*, *M. arenaria*, *M. javanica*, *Pratylenchus coffeae*, and *Rotylenchulus reniformis*; Beans & peas: *Meloidogyne* spp., *Heterodera* spp., *Belonolaimus* spp., *Helicotylenchus* spp., *Rotylenchulus reniformis*, *Paratrichodorus anemones*, and *Trichodorus* spp.; cassava: *Rotylenchulus reniformis*, *Meloidogyne* spp. cereals: *Anguina tritici* (Emmer, rye, spelt wheat), *Bidara avenae* (oat, wheat), *Ditylenchus dipsaci* (rye, oat), *Subanguina radicicola* (oat, barley, wheat, rye), *Meloidogyne naasi* (barley, wheat, rye), *Pratylenchus* spp. (oat, wheat, barley, rye), *Paratylenchus* spp. (wheat), *Tylenchorhynchus* spp. (wheat, oat); chickpea: *Heterodera cajani*, *Rotylenchulus reniformis*, *Hoplolaimus seinhorsti*, *Meloidogyne* spp., *Pratylenchus*

spp.; Citrus: *Tylenchulus semipenetrans*, *Radopholus similis*, *Radopholus citrophilus* (Florida only), *Hemicycliophora arenaria*, *Pratylenchus* spp., *Meloidogyne* spp., *Bolonolaimus longicaudatus* (Florida only),

5 *Trichodorus*, *Paratrichodorus*, *Xiphinema* spp.; clover: *Meloidogyne* spp., *Heterodera trifolii*; coconut: *Rhadinaphelenchus cocophilus*; coffee: *Meloidogyne incognita* (Most important in Brazil), *M. exigua* (widespread), *Pratylenchus coffeae*, *Pratylenchus*

10 *brachyurus*, *Radopholus similis*, *Rotylenchulus reniformis*, *Helicotylenchus* spp.; corn: *Pratylenchus* spp., *Paratrichodorus minor*, *Longidorus* spp., *Hoplolaimus columbus*; cotton: *Meloidogyne incognita*, *Belonolaimus longicaudatus*, *Rotylenchulus reniformis*, *Hoplolaimus*

15 *galeatus*, *Pratylenchus* spp., *Tylenchorhynchus* spp., *Paratrichodorus minor*; grapes: *Xiphinema* spp., *Pratylenchus vulnus*, *Meloidogyne* spp., *Tylenchulus semipenetrans*, *Rotylenchulus reniformis*; grasses: *Pratylenchus* spp., *Longidorus* spp., *Paratrichodorus*

20 *christiei*, *Xiphinema* spp., *Ditylenchus* spp.; peanut: *Pratylenchus* spp., *Meloidogyne hapla.*, *Meloidogyne arenaria*, *Criconemella* spp., *Belonolaimus longicaudatus* (in Eastern United States); pigeonpea: *Heterodera cajani*, *Rotylenchulus reniformis*, *Hoplolaimus seinhorsti*,

25 *Meloidogyne* spp., *Pratylenchus* spp.; pineapple: *Paratrichodorus christiei*, *Criconemella* spp., *Meloidogyne* spp., *Rotylenchulus reniformis*, *Helicotylenchus* spp., *Pratylenchus* spp., *Paratylenchus* spp.; potato: *Globodera rostochiensis*, *Globodera pallida*, *Meloidogyne* spp.,

30 *Pratylenchus* spp., *Trichodorus primitivus*, *Ditylenchus* spp., *Paratrichodorus* spp., *Nacoabbus aberrans*; rice: *Aphelenchiodes besseyi*, *Ditylenchus angustus*, *Hirschmanniella* spp., *Heterodera oryzae*, *Meloidogyne* spp.

35 small fruits: *Meloidogyne* spp.; *Pratylenchus* spp., *Xiphinema* spp., *Longidorus* spp., *Paratrichodorus christiei*, *Aphelenchoides* spp. (strawberry); soybean: *Heterodera glycines*, *Meloidogyne incognita*, *Meloidogyne javanica*,

-17-

*Belonolaimus* spp., *Hoplolaimus* *columbus*; sugar beet:  
*Heterodera* *schachtii*, *Ditylenchus* *dipsaci*, *Meloidogyne*  
spp., *Nacobbus* *aberrans*, *Trichodorus* spp., *Longidorus* spp.,  
5 *Paratrichodorus* spp.; sugar cane: *Meloidogyne* spp.,  
*Pratylenchus* spp., *Radopholus* spp., *Heterodera* spp.,  
*Hoplolaimus* spp., *Helicotylenchus* spp., *Scutellonema* spp.,  
*Belonolaimus* spp., *Tylenchorhynchus* spp., *Xiphinema* spp.,  
*Longidorus* spp., *Paratrichodorus* spp.; tea: *Meloidogyne*  
spp., *Pratylenchus* spp., *Radopholus* *similis*,  
10 *Hemicriconemoides* *kanayaensis*, *Helicotylenchus* spp.,  
*Paratylenchus* *curvittatus*; tobacco: *Meloidogyne* spp.,  
*Pratylenchus* spp., *Tylenchorhynchus* *claytoni*, *Globodera*  
*tabacum*, *Trichodorus* spp., *Xiphinema* *americanum*,  
*Ditylenchus* *dipsaci* (Europe only), *Paratrichodorus* spp.;  
15 tomato: *Pratylenchus* spp., *Meloidogyne* spp.; tree fruits:  
*Pratylenchus* spp. (apple, pear, stone fruits),  
*Paratylenchus* spp. (apple, pear), *Xiphinema* spp. (pear,  
cherry, peach), *Cacopaurus* *pestis* (walnut), *Meloidogyne*  
spp. (stone fruits, apple, etc.), *Longidorus* spp. (cherry),  
20 *Criconemella* spp. (peach), and *Tylenchulus* spp. (olive).

In view of the foregoing, it will be apparent  
that plants which may be employed in practicing the present  
invention include (but are not limited to) tobacco  
(*Nicotiana* *tabacum*), potato (*Solanum* *tuberosum*), soybean  
25 (*glycine* *max*), peanuts (*Arachis* *hypogaea*), cotton  
(*Gossypium* *hirsutum*), cassava (*Manihot* *esculenta*), coffee  
(*Cofea* spp.), coconut (*Cocos* *nucifera*), pineapple (*Ananas*  
*comosus*), citrus trees (*Citrus* spp.), banana (*Musa* spp.),  
corn (*Zea* *mays*), wheat, oats, rye, barley, rice, and  
30 vegetables such as green beans (*Phaseolus* *vulgaris*), lima  
beans (*Phaseolus* *limensis*), and peas (*Lathyrus* spp.).  
Thus, an illustrative category of plants which may be used  
to practice the present invention are the dicots, and a  
more particular category of plants which may be used to  
35 practice the present invention are the members of the  
family *Solanaceae*.

-18-

In practice, a crop comprising a plurality of plants of the invention are planted together in an agricultural field. By "agricultural field", we mean a common plot of soil or a greenhouse, with the determinative feature typically being that a common population of nematodes infect that crop of plants. Thus, the present invention provides a method of combatting plant parasitic nematodes in an agricultural field, by planting the field with a crop of plants according to the invention.

The examples which follow are set forth to illustrate the present invention, and are not to be construed as limiting thereof.

#### EXAMPLE 1

##### Isolation and Expression of Genomic

##### Root-Specific Clone RB7

*Nicotiana tabacum* cv Wisconsin 38 was used as the source of material for cloning and gene characterization. Genomic DNA was partially digested with *Sau3A* and size-fractionated on 5 to 20% potassium acetate gradients. Size fractions of 17 to 23 kb were pooled and ligated into the  $\lambda$  vector, EMBL3b that had been digested with *Bam*HI and *Eco*RI. See A. Frischauf et al., J. Mol. Biol. 170, 827-842 (1983). A primary library of approximately  $3.5 \times 10^6$  recombinants was screened by plaque hybridization. Positive clones were plaque purified. Restriction maps of the genomic clones were constructed using the rapid mapping procedure of Rachwitz et al., Gene 30, 195-200 (1984).

Regions encoding the root-specific clones were identified by Southern blots. To further define the transcribed regions, we took advantage of the fact that the genes are expressed at high levels. Thus, probes made of cDNA of reverse transcribed poly(A+)RNA would hybridize to Southern blots of restricted genomic clones in a manner analogous to differential screening experiments. See F. Kilcherr, Nature 321, 493-499 (1986). The clones were

-19-

digested with the appropriate restriction enzymes and the fragments separated on agarose gels. These fragments were then Southern blotted to nitrocellulose filters and probed with reverse transcribed root poly(A+)RNA. The probe was  
5 primed using random hexanucleotides (Pharmacia Biochemicals, Inc.) such that the 3' termini of the mRNA molecules would not be over represented among the probe.

Clones hybridizing to each root-specific cDNA clone were plaque purified. Comparisons of the restriction  
10 maps of the genomic clones with genomic Southern hybridization experiments (not shown) reveal a good correlation of the sequences hybridizing to the root-specific cDNA clones. Clone  $\lambda$ 5A hybridized to the cDNA clone TobRB7. This appears to be the genomic clone  
15 corresponding to TobRB7 and accordingly was designated as TobRB7-5A (SEQ ID NO:6) and used to generate the promoter sequences employed in the experiments described below. The cell membrane channel protein is set forth as SEQ ID NO:7.

## EXAMPLE 2

### 20 Identification of a Nematode-Responsive Element Within the TobRB7 Promoter

The ability of the TobRB7 promoter region of the  $\lambda$ 5A genomic clone to regulate the expression of a heterologous reporter gene was tested by cloning  
25 approximately 1.4 kb of 5' flanking sequence into pBI101.2

The length of the TobRB7 flanking region employed was varied to explore how various portions of the flanking region affected expression of GUS.

In brief, a TobRB7 5' flanking region was  
30 isolated from  $\lambda$ 5A and fused with  $\beta$ -glucuronidase in the *Agrobacterium* binary vector, pBI 101.2. This vector contains a  $\beta$ -glucuronidase (GUS) reporter gene and an *nptII* selectable marker flanked by the T-DNA border sequences (R. Jefferson et al., *EMBO J.* 6, 3901-3907 (1987)). The TobRB7  
35 structural gene was completely removed and the TobRB7 flanking regions fused to the GUS initiating methionene

-20-

codon. The construction was mobilized into an *Agrobacterium* host that carries a disarmed Ti-plasmid (LBA4404) capable of providing (in trans) the *vir* functions required for T-DNA transfer and integration into the plant genome, essentially as described by An et al., in S. Belvin and R. Schilperroot, eds., *Plant Molecular Biology Manual*, Martinus Nijhoff, Dordrecht, The Netherlands, pp A3-1-19 (1988). *Nicotiana tabacum* SR1 leaf discs were infected and transformants selected and regenerated as described by An et al., *Plant Physiol.* 81, 301-305 (1986).

Whole plants or excised root and leaf tissue were assayed for GUS expression according to Jefferson et al., *supra*. For histochemical staining, plants were incubated in the 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide (X-GLUC) at 37°C overnight. Tissues expressing GUS activity cleave this substrate and thereby stain blue. After the incubation the tissues were bleached in 70% ethanol. GUS enzyme activities were measured using the fluorogenic assay described by Jefferson et al.

The activity of the various deletion mutants was tested. The greatest root-specific gene expression was obtained with the  $\Delta$ 0.6 deletion mutant (SEQ ID NO:4). Only the  $\Delta$ 0.3 deletion mutant (SEQ ID NO:5) was inactive as a promoter, indicating that the TobRB7 promoter is found in the region extending about 800 nucleotides upstream from the TobRB7 structural gene. However, the  $\Delta$ 0.3 deletion mutant (SEQ ID NO:5) contains the RB7 nematode-responsive element, as discussed below.

### EXAMPLE 3

#### Localization of Gene Activation in Nematode Infected Plants

Transgenic tobacco plants prepared as described in Example 2 above were infected with tobacco root-knot nematodes (*Meloidogyne incognita*) in accordance with known techniques. See, e.g., C. Opperman et al., *Plant Disease*, 869-871 (October 1988). Roots were stained for GUS

-21-

activity (blue) and nematodes were stained red at three stages: (a) 24-48 hours post infection; (b) 7-10 days post infection; and (c) 20-25 days post infection. Nematodes were stained after GUS staining by incubating roots in 95% ethanol/glacial acetic acid (1:1) plus five drops of acid fushsin (per 100 mLs) for four hours, then destained in a saturated chloral hydrate solution for twelve hours to overnight.

GUS activity was generally found in the elongation zone of the root. At 24-48 hours post infection, second stage juvenile nematodes have penetrated the tobacco roots, are in the corticle tissue and are migrating in search of an appropriate feeding site. Juveniles in the vascular tissue at this stage have already begun to establish feeding sites. At 7-10 days post infection, swollen late second stage juveniles are seen with their heads in the feeding site. At 20-25 days post infection, adult nematodes are seen protruding from galled root tissue, with their head still embedded in the vascular tissue and the posterior exposed to allow egg deposition.

GUS activity in nematode infected root tissue of plants transformed with the various deletion mutants described in Example 2 indicated that the nematode-responsive element of the TobRB7 promoter is located in the A0.3 (SEQ ID NO:5) deletion mutant.

Similar results are obtained with the peanut root-knot nematode (*Meloidogyne arenaria*).

During the foregoing experiments, it was observed that duration of gene expression in nematode-infected plants was much longer than in uninfected plants, and that the regions of gene activity were no longer restricted to the elongation zone of the root. For example, in each location where a nematode was able to establish a feeding site, gene expression continued at that site for as long as 25-30 days (i.e., the duration of the nematode life cycle).



-22-

**EXAMPLE 4****Inhibition of Nematode Feeding Site Formation  
by Expression of Sense or Antisense TobRB7 mRNA**

This example demonstrates the ability of transgenic plants expressing sense and anti-sense TobRB7 mRNA under the control of a constitutively active promoter to interfere with the establishment of root-knot nematode feeding sites. The constructions employed are described in Figure 1, and the plants were prepared in essentially the same manner as described in Example 2 above. The sense DNA employed had the sequence given herein as SEQ ID NO:1, and the antisense DNA employed had the sequence given herein as SEQ ID NO:3. The promoter employed was the Cauliflower Mosaic Virus 35S promoter, and the termination signal employed was the nos terminator. The constructs were transferred to the *Agrobacterium* binary vector pBIN19 and transgenic plants were produced in essentially the same manner as described above: tobacco leaf disks were transformed and transformants selected on kanamycin; regenerants were allowed to self and set seeds; seeds (R2) were germinated on kanamycin and segregation of the Kan<sup>r</sup> marker assayed; those plants exhibiting a 3:1 segregation (i.e., containing a single locus of integration) were allowed to self; progeny of the R2 were germinated on kanamycin to determine those R2 progeny that were homozygous for the transgene.

The phenotypes of a large number of control, sense, and antisense plants were examined. Control plants looked like normal tobacco. Sense and antisense plants exhibited similar phenotypes: 1) long internodes, (2) narrow and pointed leaves, and (3) early flowering. These phenotypes resemble "stress" phenotypes exhibited by plants grown in suboptimal conditions, such as small pots. It appears that the "stress" phenotype in sense plants results from the phenomenon of co-suppression: a phenomenon in which plants carrying transgenes in the sense orientation show reduced, rather than increased, levels of gene

-23-

expression. See, e.g., C. Napoli et al., *The Plant Cell* 2, 279-289 (1990).

Transgenic plants of sense transformants, anti-sense transformants, and control transformants were infected with second-stage juveniles of *M. arenaria* in essentially the same manner as described above. Approximately 100,000 nematodes suspended in sterile water were pipetted along the roots of plants growing on agar plates. Plants were maintained in a growth chamber at 25°C. At 24 hr post infection, juveniles were observed in various stages of root penetration on all plates. Galls were visible on all treatments by 3-5 days post infection.

Roots were harvested from plates 2A, 2B, and 7 (anti-sense); 13 and 37 (sense); and 22A and 22B (control) at 21 days post-infection. Initial observations revealed substantial and extensive galling of the sense and control plants. Galls often appeared in clusters along the root. It appeared that in a number of galls, adult female nematodes had begun reproduction. In contrast, few galls were present on the anti-sense plants. Those that were present occurred singly rather than in clusters and were substantially reduced in size compared to the sense and control plants (<50% the diameter). Two of the three plates yielded no plants with visible galling at 21 days post-infection.

Roots from each treatment were stained with acid fuchsin to determine stage of nematode development and the degree of root penetration. Roots of sense and control plants were infected with numerous nematodes in various stages of development. Mature females were observed in several galls and egg production appeared to have been initiated. Galls contained numerous nematodes. Other stages observed included vermiform second-stage juveniles, swollen second-stage juveniles, and third/fourth stage juveniles. No adult males were observed within roots or on plates. Far fewer nematodes were observed in anti-sense plants. Those that were present were mostly vermiform or

-24-

swollen second-stage juveniles. No adult female nematodes were found. Several adult male nematodes were observed within the roots, but not on the plate surface. Galls that were present generally contained a single nematode and tended to occur at root junctions.

### EXAMPLE 5

#### Effect on Nematode Nematode Egg Mass Rating of Expression of Sense or Antisense TobRB7 mRNA under The Control of a Constitutive Promoter

Transgenic tobacco plants expressing sense or antisense TobRB7 mRNA prepared as described above were infected with tobacco root-knot nematodes (*Meloidogyne incognita*) in accordance with known techniques. See, e.g., C. Opperman et al., *Plant Disease*, 869-871 (October 1988). 63 days after infection, roots were harvested, egg masses were stained with Phloxine B to facilitate counting in accordance with known techniques and egg masses counted. Both sense and antisense plants were found resistant to nematodes. These data are given in Table 1 below.

TABLE 1: Egg Mass Ratings at 63 Days After Infection

Transformant Line	Egg Mass Rating	Number of Eggs	Plant Type
37	2.6±0.5	1120	sense
6	3.6±1.0	3516	antisense
20	3.8±1.3	3270	antisense
2	4.0±1.0	NA	antisense
13	4.3±0.5	5400	sense
34	4.4±0.7	4594	sense
36	4.5±0.8	6980	sense
21	4.6±0.5	5300	control
22	4.7±0.5	6000	control

Egg Mass Rating: 0=no egg masses; 1=<10 egg masses; 2=10-50 egg masses; 3=50-150 egg masses; 4=150-300 egg masses; 5=>300 egg masses. NA=not available.

-25-

## EXAMPLE 6

Inhibition of Nematode Feeding Site Formation by  
Expression of Sense or Antisense TobRB7 mRNA under  
The Control of a Nematode-Responsive Element  
or a Root-Specific Gene Promoter

5 Transgenic plants expressing sense anti-sense  
TobRB7 mRNA under the control of a promoter comprising a  
root specific gene promoter or a nematode-responsive  
10 element interfere with the establishment of root-knot  
nematode feeding sites. The constructions employed are  
described in Figure 2. Sense, antisense, and control  
plants were produced in essentially the same manner as  
described in Example 4 above, except that the root specific  
15 promoter described above and having the sequence given in  
SEQ ID NO:4 was employed in place of the CaMV 35S promoter.  
Additionally, sense, antisense, and control plants were  
produced in essentially the same manner as described in  
20 Example 4 above, except that the nematode-responsive  
element described above and having the sequence given  
herein as SEQ ID NO:5 was employed in place of the CaMV 35S  
promoter. Resistance to nematodes is shown in the same  
manner as described above.

The foregoing examples are illustrative of the  
present invention, and are not to be construed as limiting  
25 thereof. The invention is defined by the following claims,  
with equivalents of the claims to be included therein.

-26-

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Conkling, Mark A.  
Opperman, Charles H.  
Acedo, Gregoria N.  
Song, Wen

(ii) TITLE OF INVENTION: Nematode Resistant Transgenic Plants

(iii) NUMBER OF SEQUENCES: 7

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Kenneth D. Sibley; Bell, Seltzer, Park and  
Gibson  
(B) STREET: Post Office Drawer 34009  
(C) CITY: Charlotte  
(D) STATE: North Carolina  
(E) COUNTRY: U.S.A.  
(F) ZIP: 28234

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Sibley, Kenneth D.  
(B) REGISTRATION NUMBER: 31,665  
(C) REFERENCE/DOCKET NUMBER: 5051-201

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 919-881-3140  
(B) TELEFAX: 919-881-3175  
(C) TELEX: 575102

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 938 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

-27-

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 47..799

## (ix) FEATURE:

(A) NAME/KEY: mat\_peptide

(B) LOCATION: 47..796

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTTAAATTGA GCTTCTTTTG GGGCATTTTT CTAGTGAGAA CTAAAA ATG GTG AGG	55
Met Val Arg	
1	
ATT GCC TTT GGT AGC ATT GGT GAC TCT TTT AGT GTT GGA TCA TTG AAG	103
Ile Ala Phe Gly Ser Ile Gly Asp Ser Phe Ser Val Gly Ser Leu Lys	
5 10 15	
GCC TAT GTA GCT GAG TTT ATT GCT ACT CTT CTC TTT GTG TTT GCT GGG	151
Ala Tyr Val Ala Glu Phe Ile Ala Thr Leu Leu Phe Val Phe Ala Gly	
20 25 30 35	
GTT GGG TCT GCT ATA GCT TAT AAT AAA TTG ACA GCA GAT GCA GCT CTT	199
Val Gly Ser Ala Ile Ala Tyr Asn Lys Leu Thr Ala Asp Ala Ala Leu	
40 45 50	
GAT CCA GCT GGT CTA GTA GCA GTA GCT GTG GCT CAT GCA TTT GCA TTG	247
Asp Pro Ala Gly Leu Val Ala Val Ala Val Ala His Ala Phe Ala Leu	
55 60 65	
TTT GTT GGG GTT TCC ATA GCA GCC AAT ATT TCA GGT GGC CAT TTG AAT	295
Phe Val Gly Val Ser Ile Ala Ala Asn Ile Ser Gly Gly His Leu Asn	
70 75 80	
CCA GCT GTC ACT TTG GGA TTG GCT GTT GGT GGA AAC ATC ACC ATC TTG	343
Pro Ala Val Thr Leu Gly Leu Ala Val Gly Gly Asn Ile Thr Ile Leu	
85 90 95	
ACT GGC TTC TTC TAC TGG ATT GCC CAA TTG CTT GGC TCC ACA GTT GCT	391
Thr Gly Phe Phe Tyr Trp Ile Ala Gln Leu Leu Gly Ser Thr Val Ala	
100 105 110 115	
TGC CTC CTC CTC AAA TAC GTT ACT AAT GGA TTG GCT GTT CCA ACC CAT	439
Cys Leu Leu Leu Lys Tyr Val Thr Asn Gly Leu Ala Val Pro Thr His	
120 125 130	
GGA GTT GCT GCT GGG CTC AAT GGA TTA CAA GGA GTG GTG ATG GAG ATA	487
Gly Val Ala Ala Gly Leu Asn Gly Leu Gln Gly Val Val Met Glu Ile	
135 140 145	
ATC ATA ACC TTT GCA CTG GTC TAC ACT GTT TAT GCA ACA GCA GCA GAC	535
Ile Ile Thr Phe Ala Leu Val Tyr Thr Val Tyr Ala Thr Ala Ala Asp	
150 155 160	

-28-

CCT AAA AAG GGC TCA CTT GGA ACC ATT GCA CCC ATT GCA ATT GGG TTC	583
Pro Lys Lys Gly Ser Leu Gly Thr Ile Ala Pro Ile Ala Ile Gly Phe	
165 170 175	
ATT GTT GGG GCC AAC ATT TTG GCA GCT GGT CCA TTC AGT GGT GGG TCA	631
Ile Val Gly Ala Asn Ile Leu Ala Ala Gly Pro Phe Ser Gly Gly Ser	
180 185 190 195	
ATG AAC CCA GCT CGA TCA TTT GGG CCA GCT GTG GTT GCA GGA GAC TTT	679
Met Asn Pro Ala Arg Ser Phe Gly Pro Ala Val Val Ala Gly Asp Phe	
200 205 210	
TCT CAA AAC TGG ATC TAT TGG GCC GGC CCA CTC ATT GGT GGA GGA TTA	727
Ser Gln Asn Trp Ile Tyr Trp Ala Gly Pro Leu Ile Gly Gly Gly Leu	
215 220 225	
GCT GGG TTT ATT TAT GGA GAT GTC TTT ATT GGA TGC CAC ACC CCA CTT	775
Ala Gly Phe Ile Tyr Gly Asp Val Phe Ile Gly Cys His Thr Pro Leu	
230 235 240	
CCA ACC TCA GAA GAC TAT GCT TAAACTTAA AAGAAGACAA GTCTGTCTTC	826
Pro Thr Ser Glu Asp Tyr Ala	
245 250	
AATGTTTCTT TGTGTGTTTT CAAATGCAAT GTTGATTTTT AATTTAAGCT TTGTATATTA	886
TGCTATGCAA CAAGTTTGTT TCCAATGAAA TATCATGTTT TGGTTTCTTT TG	938

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 250 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Val Arg Ile Ala Phe Gly Ser Ile Gly Asp Ser Phe Ser Val Gly	
1 5 10 15	
Ser Leu Lys Ala Tyr Val Ala Glu Phe Ile Ala Thr Leu Leu Phe Val	
20 25 30	
Phe Ala Gly Val Gly Ser Ala Ile Ala Tyr Asn Lys Leu Thr Ala Asp	
35 40 45	
Ala Ala Leu Asp Pro Ala Gly Leu Val Ala Val Ala Val Ala His Ala	
50 55 60	
Phe Ala Leu Phe Val Gly Val Ser Ile Ala Ala Asn Ile Ser Gly Gly	
65 70 75 80	

-29-

His Leu Asn Pro Ala Val Thr Leu Gly Leu Ala Val Gly Gly Asn Ile  
                     85                    90                    95  
 Thr Ile Leu Thr Gly Phe Phe Tyr Trp Ile Ala Gln Leu Leu Gly Ser  
                     100                    105                    110  
 Thr Val Ala Cys Leu Leu Leu Lys Tyr Val Thr Asn Gly Leu Ala Val  
                     115                    120                    125  
 Pro Thr His Gly Val Ala Ala Gly Leu Asn Gly Leu Gln Gly Val Val  
                     130                    135                    140  
 Met Glu Ile Ile Ile Thr Phe Ala Leu Val Tyr Thr Val Tyr Ala Thr  
                     145                    150                    155                    160  
 Ala Ala Asp Pro Lys Lys Gly Ser Leu Gly Thr Ile Ala Pro Ile Ala  
                     165                    170                    175  
 Ile Gly Phe Ile Val Gly Ala Asn Ile Leu Ala Ala Gly Pro Phe Ser  
                     180                    185                    190  
 Gly Gly Ser Met Asn Pro Ala Arg Ser Phe Gly Pro Ala Val Val Ala  
                     195                    200                    205  
 Gly Asp Phe Ser Gln Asn Trp Ile Tyr Trp Ala Gly Pro Leu Ile Gly  
                     210                    215                    220  
 Gly Gly Leu Ala Gly Phe Ile Tyr Gly Asp Val Phe Ile Gly Cys His  
                     225                    230                    235                    240  
 Thr Pro Leu Pro Thr Ser Glu Asp Tyr Ala  
                     245                    250

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 938 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: YES

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAAAAGAAAC CAAACATGA TATTCATTG GAAACAACT TGTTCATAG CATAATATAC	60
AAAGCTTAAA TTAATAATCA ACATTGCATT TGAAACACA CAAAGAAACA TTGAAGACAG	120
ACTTGTCTTC TTTAAGTTT TAAGCATAGT CTTCTGAGGT TGGAAGTGGG GTGTGGCATC	180



-30-

CAATAAAGAC ATCTCCATAA ATAAACCCAG CTAATCCTCC ACCAATGAGT GGGCCGGCCC	240
AATAGATCCA GTTTGTAGAA AAGTCTCCTG CAACCACAGC TGGCCCAAAT GATCGAGCTG	300
GGTTCATTGA CCCACCACTG AATGGACCAG CTGCCAAAAT GTTGGCCCCA ACAATGAACC	360
CAATTGCAAT GGGTGCAATG GTTCCAAGTG AGCCCTTTTT AGGGTCTGCT GCTGTTGCAT	420
AAACAGTGTA GACCAGTGCA AAGGTTATGA TTATCTCCAT CACCACTCCT TGTAATCCAT	480
TGAGCCCAGC AGCAACTCCA TGGGTTGGAA CAGCCAATCC ATTAGTAACG TATTTGAGGA	540
GGAGGCAAGC AACTGTGGAG CCAAGCAATT GGGCAATCCA GTAGAAGAAG CCAGTCAAGA	600
TGGTGATGTT TCCACCAACA GCCAATCCCA AAGTGACAGC TGGATTCAA TGGCCACCTG	660
AAATATTGGC TGCTATGGAA ACCCCAACAA ACAATGCAAA TGCATGAGCC ACAGCTACTG	720
CTACTAGACC AGCTGGATCA AGAGCTGCAT CTGCTGTCAA TTTATTATAA GCTATAGCAG	780
ACCCAACCCC AGCAAACACA AAGAGAAGAG TAGCAATAAA CTCAGCTACA TAGGCCTTCA	840
ATGATCCAAC ACTAAAAGAG TCACCAATGC TACCAAAGGC AATCCTCACC ATTTTITAGTT	900
CTCACTAGAA AATGCCCCA AAAGAAGCTC AATTTAAG	938

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 706 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTCTACACA ATGTGAATTT GAATTAGTTT GGTCATACGG TATATCATAT GATTATAAAT	60
AAAAAAAATT AGCAAAAGAA TATAATTTAT TAAATATTTT ACACCATACC AAACACAACC	120
GCATTATATA TAATCTTAAT TATCATTATC ACCAGCATCA ACATTATAAT GATTCCCCTA	180
TGCGTTGGAA CGTCATTATA GTTATTCTAA ACAAGAAAGA AATTTGTTCT TGACATCAGA	240
CATCTAGTAT TATAACTCTA GTGGAGCTTA CCTTTTCTTT TCCTTCTTTT TTTTCTTCTT	300
AAAAAAATTA TCACTTTTTA AATCTTGAT ATTAGTTAAG CTTATCTAAA CAAAGTTTTA	360
AATTCATTTT TTAACGTCC ATTACAATGT AATATAACTT AGTCGTCTCA ATTAACCAT	420
TAATGTGAAA TATAAATCAA AAAAAGCCAA AGGGCGGTGG GACGGCGCCA ATCATTGTGTC	480

-31-

CTAGTCCACT CAAATAAGGC CCATGGTCGG CAAAACCAA CACAAAATGT GTTATTTTTA 540  
 ATTTTTCCT CTTTATTGT TAAAGTTGCA AAATGTGTTA TTTTGGTAA GACCCTATGG 600  
 ATATATAAAG ACAGGTTATG TGAAACTTGG AAAACCATCA AGTTTAAAGC AAAACCCTCT 660  
 TAAGAACTTA AATTGAGCTT CTTTGGGGC ATTTTCTAG TGAGAA 706

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 368 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGCTTATCTA AACAAAGTTT TAAATTCATT TCTTAAACGT CCATTACAAT GTAATATAAC 60  
 TTAGTCGTCT CAATTAAACC ATTAATGTGA AATATAATC AAAAAAGCC AAAGGGCGGT 120  
 GGGACGGCGC CAATCATTTG TCCTAGTCCA CTCAAATAAG GCCCATGGTC GGCAAAACCA 180  
 AACACAAAAT GTGTTATTTT TAATTTTTC CTCTTTTATT GTTAAAGTTG CAAAATGTGT 240  
 TATTTTGGT AAGACCCTAT GGATATATAA AGACAGGTTA TGTGAACTT GGAAAACCAT 300  
 CAAGTTTAA GCAAAACCCT CTTAAGAACT TAAATTGAGC TTCTTTGGG GCATTTTCT 360  
 AGTGAGAA 368

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 3426 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: promoter  
 (B) LOCATION: 1..1877

## (ix) FEATURE:

- (A) NAME/KEY: exon  
 (B) LOCATION: 1954..2079

## (ix) FEATURE:

-32-

(A) NAME/KEY: intron  
(B) LOCATION: 2080..2375

(ix) FEATURE:  
(A) NAME/KEY: exon  
(B) LOCATION: 2376..2627

(ix) FEATURE:  
(A) NAME/KEY: intron  
(B) LOCATION: 2628..2912

(ix) FEATURE:  
(A) NAME/KEY: exon  
(B) LOCATION: 2913..3284

(ix) FEATURE:  
(A) NAME/KEY: 5'UTR  
(B) LOCATION: 1878..1953

(ix) FEATURE:  
(A) NAME/KEY: CDS  
(B) LOCATION: join(1954..2079, 2376..2627, 2913..3284)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGATCCCCCT CTTTATAAT AGAGGGTCAT TACTTTATTT ACAATAAAAT AATAAAATAA	60
AGCATATAGT GGAGGACCCA TGATGACTTG TTTCTTCCTC GATTTTCGCC GAGATTCTCT	120
CCCATAGTGC GGTGCAACG GCCCTTGTCT GCGAGCTCGA TACTGGTTCG AGCTCGGCAT	180
TGGACCGAGC CCTCGACCTT GGTCCGAGCT CGATTCTGAC TTGGGGTCTC GGTATTCGGG	240
GTGAGTGTTG GTCGGTCTAT GCATCTTCGA TAATCTCCGT TTTGCCTCGT AGTTCGATTT	300
GGATATGAGC TCGATAATGA TACCGAGCTT GTCATTGATC GGTCTTAGAG CTCGAAGTTC	360
GACGCCTTTA CTTCGGACCT TGACCGAGCT TGTTATGTAG ATATCCTTTG ATCGAAACAT	420
TATCGTTTTG ACCAATCCGT ACGACTGACT CAAATCGATT TGACCGCACA CAAGATTATT	480
TTCGAAAGAC CCTCGACGTC TTGGAGTATA AAATAATTTA GTAAAGAGAG TAATTGTTCCG	540
TTAAAAATCT TGACACCATT CCAAGCATAC CCCTTATTGT ACTTCAATTA ATTATCATT	600
TATCAGCATA AACATTATAA TAAGTTTCTT GCGTGTTGGA ACGTCATTTT AGTTATTCTA	660
AAGAGGAAAT AGTTTCTTTT TTGCTCATGA CATCAGACAT CTGGACTACT ATACTGGAGT	720
TTACCTTTTC TTCTCCTCTT TTTCTTATTG TTCCTCTAAA AAAAATTATC ACTTTTAA	780
TGCATTAGTT AAACCTATCT CAACAACGTT TAAAATTCAT TTCTTGAATG CCCATTACAA	840
TGTAATAGTA TAACTTAATT AGTCGTCTCC ATGAACCATT AATACGTACG GAGTAATATA	900

-33-

AAACACCATT GGGGAGTTCA ATTTGCAATA ATTTCTTGCA AAAATGTAAA GTACCTTTTT	960
GTTCTTGCAA AATTTTACAA ATAAAAATTT GCAGCTCTTT TTTTCTCTC TCTCCAAATA	1020
CTAGCTCAAA ACCCACAAAT ATTTTGAAT TTATGGCATA CTTTGAAT GCGTTGATG	1080
CAACTATTTT CCTTTAGGAA ATATTCACAA CAATCTAAGA CAATCAAAAA GTAGAAAATA	1140
GTTTGTAAAA AGGGATGTGG AGGACATCTT AATCAAATAT TTTCAGTTTA AAACCTTGAAA	1200
ATGAAAAAAC ACCCGAAAGG AAATGATTCG TTCTTTAATA TGCCTACAC AATGTGAATT	1260
TGAATTAGTT TGGTCATACG GTATATCATA TGATTATAAA TAAAAAAAT TAGCAAAAGA	1320
ATATAATTTA TTAAATATTT TACACCATAC CAAACACAAC CGCATTATAT ATAATCTTAA	1380
TTATCATTAT CACCAGCATC AACATTATAA TGATTCCCCT ATGCGTTGGA ACGTCATTAT	1440
AGTTATTCTA AACAAGAAAG AAATTTGTTT TTGACATCAG ACATCTAGTA TTATAACTCT	1500
AGTGGAGCTT ACCTTTTCTT TTCCTTCTTT TTTTCTTCT TAAAAAATT ATCACTTTTT	1560
AAATCTTGTA TATTAGTTAA GCTTATCTAA ACAAAGTTTT AAATTCATTT CTAAACGTC	1620
CATTACAATG TAATATAACT TAGTCGCTC AATTAAACCA TTAATGTGAA ATATAATCA	1680
AAAAAAGCCA AAGGGCGGTG GGACGGCGCC AATCATTTGT CCTAGTCCAC TCAAATAAGG	1740
CCCATGGTCG GCAAACCAA ACACAAAATG TGTTATTTTT AATTTTTTCC TCTTTTATTG	1800
TTAAAGTTGC AAAATGTGTT ATTTTGGTA AGACCCTATG GATATATAAA GACAGGTAT	1860
GTGAAACTTG GAAACCATC AAGTTTAAAG CAAAACCTC TTAAGAACTT AAATTGAGCT	1920
TCTTTTGGGG CATTTTCTA GTGAGAACTA AAA ATG GTG AGG ATT GCC TTT GGT	1974
Met Val Arg Ile Ala Phe Gly	
1 5	
AGC ATT GGT GAC TCT TTT AGT GTT GGA TCA TTG AAG GCC TAT GTA GCT	2022
Ser Ile Gly Asp Ser Phe Ser Val Gly Ser Leu Lys Ala Tyr Val Ala	
10 15 20	
GAG TTT ATT GCT ACT CTT CTC TTT GTG TTT GCT GGG GTT GGG TCT GCT	2070
Glu Phe Ile Ala Thr Leu Leu Phe Val Phe Ala Gly Val Gly Ser Ala	
25 30 35	
ATA GCT TAT AGTAAGTAAC ACTTCTCTAA TTAACTTGC ATGCTAACAT	2119
Ile Ala Tyr	
40	
AAATACTTAA TCTGCTCTAG CACTAAATAG TAAAAAGAGC AATCAGGTGC ACTAAGGTCC	2179
CATTAATTCG TTATGCACAT GCCACGGAGT CTAGAGAAAG ACTAGACTGG CTCTATCATA	2239
TTCAATTTTA CCTTACATTT TACTAGATGC CGTTTTCTCA ATCCATAACC GAAAACAACA	2299

-34-

TAACTTTTAC AGTTACACCA AGACTGCCTA ATTAACCTTT TTTTTTTTTT TTTTGGCTTT	2359
GTGGGGTGAT TTTGTA GAT AAA TTG ACA GCA GAT GCA GCT CTT GAT CCA Asp Lys Leu Thr Ala Asp Ala Ala Leu Asp Pro 45 50	2408
GCT GGT CTA GTA GCA GTA GCT GTG GCT CAT GCA TTT GCA TTG TTT GTT Ala Gly Leu Val Ala Val Ala Val Ala His Ala Phe Ala Leu Phe Val 55 60 65	2456
GGG GTT TCC ATA GCA GCC AAT ATT TCA GGT GGC CAT TTG AAT CCA GCT Gly Val Ser Ile Ala Ala Asn Ile Ser Gly Gly His Leu Asn Pro Ala 70 75 80 85	2504
GTA ACT TTG GGA TTG GCT GTT GGT GGA AAC ATC ACC ATC TTG ACT GGC Val Thr Leu Gly Leu Ala Val Gly Gly Asn Ile Thr Ile Leu Thr Gly 90 95 100	2552
TTC TTC TAC TGG ATT GCC CAA TTG CTT GGC TCC ACA GTT GCT TGC CTC Phe Phe Tyr Trp Ile Ala Gln Leu Leu Gly Ser Thr Val Ala Cys Leu 105 110 115	2600
CTC CTC AAA TAC GTT ACT AAT GGA TTG GTATGTACTG CTATCATTTT Leu Leu Lys Tyr Val Thr Asn Gly Leu 120 125	2647
CAATCCATAT TATATGTCTT TTTATATTTT TCACAACCTC AATAAAAAAA CAACTTTACC	2707
TAAGACCAGC CTAAGCCGTC GTATAGCCGT CCATCCAACC CTTTAAATTA AAAAGAGCCG	2767
GCATAGTCAT AATATATGTA TATTTTCATGT AGAATATTTG TATAATTAGT GTATATTGTA	2827
CGTATATCGA CTAGAAAAAA ATAAATAATG AATATGACTG TTTATTTGTA ATTGGAGTTG	2887
GGCCTCATAT GTTGGTTTTT GGCAG GCT GTT CCA ACC CAT GGA GTT GCT GCT Ala Val Pro Thr His Gly Val Ala Ala 130 135	2939
GGG CTC AAT GGA TTA CAA GGA GTG GTG ATG GAG ATA ATC ATA ACC TTT Gly Leu Asn Gly Leu Gln Gly Val Val Met Glu Ile Ile Ile Thr Phe 140 145 150	2987
GCA CTG GTC TAC ACT GTT TAT GCA ACA GCA GCA GAC CCT AAA AAG GGC Ala Leu Val Tyr Thr Val Tyr Ala Thr Ala Ala Asp Pro Lys Lys Gly 155 160 165	3035
TCA CTT GGA ACC ATT GCA CCC ATT GCA ATT GGG TTC ATT GTT GGG GCC Ser Leu Gly Thr Ile Ala Pro Ile Ala Ile Gly Phe Ile Val Gly Ala 170 175 180	3083
AAC ATT TTG GCA GCT GGT CCA TTC AGT GGT GGG TCA ATG AAC CCA GCT Asn Ile Leu Ala Ala Gly Pro Phe Ser Gly Gly Ser Met Asn Pro Ala 185 190 195	3131

-35-

CGA TCA TTT GGG CCA GCT GTG GTT GCA GGA GAC TTT TCT CAA AAC TGG	3179
Arg Ser Phe Gly Pro Ala Val Val Ala Gly Asp Phe Ser Gln Asn Trp	
200 205 210 215	
ATC TAT TGG GCC GGC CCA CTC ATT GGT GGA GGA TTA GCT GGG TTT ATT	3227
Ile Tyr Trp Ala Gly Pro Leu Ile Gly Gly Gly Leu Ala Gly Phe Ile	
220 225 230	
TAT GGA GAT GTC TTT ATT GGA TGC CAC ACC CCA CTT CCA ACC TCA GAA	3275
Tyr Gly Asp Val Phe Ile Gly Cys His Thr Pro Leu Pro Thr Ser Glu	
235 240 245	
GAC TAT GCT TAAACTTAA AAGAAGACAA GTCTGTCTTC AATGTTTCTT	3324
Asp Tyr Ala	
250	
TGTGTGTTTT CAAATGCAAT GTTGATTTTT AATTAAAGCT TTGTATATTA TGCTATGCAA	3384
CAAGTTTGTT TCCAATGAAA TATCATGTTT TGGTTTCTTT TG	3426

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 250 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met Val Arg Ile Ala Phe Gly Ser Ile Gly Asp Ser Phe Ser Val Gly	
1 5 10 15	
Ser Leu Lys Ala Tyr Val Ala Glu Phe Ile Ala Thr Leu Leu Phe Val	
20 25 30	
Phe Ala Gly Val Gly Ser Ala Ile Ala Tyr Asp Lys Leu Thr Ala Asp	
35 40 45	
Ala Ala Leu Asp Pro Ala Gly Leu Val Ala Val Ala Val Ala His Ala	
50 55 60	
Phe Ala Leu Phe Val Gly Val Ser Ile Ala Ala Asn Ile Ser Gly Gly	
65 70 75 80	
His Leu Asn Pro Ala Val Thr Leu Gly Leu Ala Val Gly Gly Asn Ile	
85 90 95	
Thr Ile Leu Thr Gly Phe Phe Tyr Trp Ile Ala Gln Leu Leu Gly Ser	
100 105 110	
Thr Val Ala Cys Leu Leu Leu Lys Tyr Val Thr Asn Gly Leu Ala Val	
115 120 125	

-36-

Pro Thr His Gly Val Ala Ala Gly Leu Asn Gly Leu Gln Gly Val Val  
 130 135 140  
 Met Glu Ile Ile Ile Thr Phe Ala Leu Val Tyr Thr Val Tyr Ala Thr  
 145 150 155 160  
 Ala Ala Asp Pro Lys Lys Gly Ser Leu Gly Thr Ile Ala Pro Ile Ala  
 165 170 175  
 Ile Gly Phe Ile Val Gly Ala Asn Ile Leu Ala Ala Gly Pro Phe Ser  
 180 185 190  
 Gly Gly Ser Met Asn Pro Ala Arg Ser Phe Gly Pro Ala Val Val Ala  
 195 200 205  
 Gly Asp Phe Ser Gln Asn Trp Ile Tyr Trp Ala Gly Pro Leu Ile Gly  
 210 215 220  
 Gly Gly Leu Ala Gly Phe Ile Tyr Gly Asp Val Phe Ile Gly Cys His  
 225 230 235 240  
 Thr Pro Leu Pro Thr Ser Glu Asp Tyr Ala  
 245 250

**THAT WHICH IS CLAIMED IS:**

1. A DNA construct comprising a transcription cassette, which construct comprises, in the 5' to 3' direction, a promoter operable in a plant cell, and a DNA comprising at least 15 nucleotides of a DNA encoding a nematode-inducible transmembrane pore protein in either (a) the opposite orientation for expression or (b) the proper orientation for expression.

2. A DNA construct according to claim 1, which DNA encoding a nematode-inducible transmembrane pore protein is selected from the group consisting of:

(a) isolated DNA having the sequence given herein as SEQ ID NO:1 or SEQ ID NO:6;

(b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a nematode inducible transmembrane pore protein; and

(c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encode a nematode-inducible transmembrane pore protein.

3. A DNA construct according to claim 1, which DNA comprising at least 15 nucleotides of a DNA encoding a nematode-inducible transmembrane pore protein is a sense DNA in the proper orientation for expression.

4. A DNA construct according to claim 1, which DNA comprising at least 15 nucleotides of a DNA encoding a nematode-inducible transmembrane pore protein is an antisense DNA in the opposite orientation for expression.

5. A DNA construct according to claim 4, which antisense DNA includes an intron-exon junction.



-38-

6. A DNA construct according to claim 4, which antisense DNA has the sequence given herein as SEQ ID NO:3.

7. A DNA construct according to claim 1, which promoter is constitutively active in plant cells.

8. A DNA construct according to claim 1, which promoter is selectively active in plant root tissue cells.

9. A DNA construct according to claim 1, which promoter is a Cauliflower Mosaic Virus 35S promoter.

10. A DNA construct according to claim 1, which promoter is activated by a plant-parasitic nematode.

11. A DNA construct according to claim 1, which promoter is a nematode-responsive element selected from the group consisting of:

(i) isolated DNA having the sequence given herein as SEQ ID NO:5; and

(ii) isolated DNA which hybridizes to isolated DNA of (i) above and which encodes a nematode responsive element.

12. A DNA construct according to claim 1, which promoter is an RB7 nematode-responsive element.

13. A DNA construct according to claim 1 carried by a plant transformation vector.

14. A DNA construct according to claim 1 carried by a plant transformation vector, which plant transformation vector is an *Agrobacterium tumefaciens* vector.

15. A nematode-resistant transgenic plant comprising plant cells containing a DNA construct comprising a transcription cassette, which construct comprises, in the 5' to 3' direction, a promoter operable in said plant cells, and a DNA comprising at least 15 nucleotides of a DNA sequence encoding a nematode-inducible transmembrane pore protein in either (a) the opposite orientation for expression or (b) the proper orientation for expression.

16. A plant according to claim 15, which plant is a dicot.

17. A plant according to claim 15, which plant is a dicot selected from the group consisting of tobacco, potato, soybean, peanuts, pineapple, and cotton.

18. A plant according to claim 15, which plant is a member of the family Solanaceae.

19. A plant according to claim 15, which DNA sequence encoding a nematode-inducible transmembrane pore protein is selected from the group consisting of:

(a) isolated DNA having the sequence given herein as SEQ ID NO:1 or SEQ ID NO:6;

(b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a nematode inducible transmembrane pore protein; and

(c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encode a nematode-inducible transmembrane pore protein.

20. A plant according to claim 15, which DNA comprising at least 15 nucleotides of a DNA encoding a nematode-inducible transmembrane pore protein is a sense DNA in the proper orientation for expression.

21. A plant according to claim 15, which DNA comprising at least 15 nucleotides of a DNA encoding a nematode-inducible transmembrane pore protein is an antisense DNA in the opposite orientation for expression.

22. A plant according to claim 15, which promoter is constitutively active in plant cells.

23. A plant according to claim 15, which promoter is selectively active in plant root tissue cells.

24. A plant according to claim 15, which promoter is activated by a plant-parasitic nematode.

25. A plant according to claim 15, which promoter is a nematode-responsive element selected from the group consisting of:

(i) isolated DNA having the sequence given herein as SEQ ID NO:5; and

(ii) isolated DNA which hybridizes to isolated DNA of (i) above and which encodes a nematode responsive element.

26. A crop comprising a plurality of plants according to claim 15 planted together in an agricultural field.

27. A method of combatting a plant parasitic nematodes in an agricultural field, comprising planting the field with a crop of plants according to claim 15.

-41-

28. A method of making a recombinant pathogen-resistant plant, said method comprising:

providing a plant cell capable of regeneration;  
transforming said plant cell with a DNA construct comprising a transcription cassette, which construct comprises, in the 5' to 3' direction, a promoter operable in said plant cell, and a DNA comprising at least 15 nucleotides of a DNA sequence encoding a nematode-inducible transmembrane pore protein in either (a) the opposite orientation for expression or (b) the proper orientation for expression; and then

regenerating a recombinant nematode-resistant plant from said transformed plant cell.

29. A method according to claim 28, wherein said plant cell resides in a plant tissue capable of regeneration.

30. A method according to claim 28, wherein said transforming step is carried out by bombarding said plant cell with microparticles carrying said transcription cassette.

31. A method according to claim 28, wherein said transforming step is carried out by infecting said cells with an *Agrobacterium tumefaciens* containing a Ti plasmid carrying said transcription cassette.

32. A DNA construct comprising a transcription cassette, which construct comprises, in the 5' to 3' direction, a promoter operable in a plant cell, and a DNA encoding an enzymatic RNA molecule directed against the mRNA transcript of a DNA sequence encoding a nematode-inducible transmembrane pore protein.

33. A DNA construct according to claim 32, which DNA sequence encoding a nematode-inducible transmembrane pore protein is selected from the group consisting of:

(a) isolated DNA having the sequence given herein as SEQ ID NO:1 or SEQ ID NO:6;

(b) isolated DNA which hybridizes to isolated DNA of (a) above and which encodes a nematode inducible transmembrane pore protein; and

(c) isolated DNA differing from the isolated DNAs of (a) and (b) above in nucleotide sequence due to the degeneracy of the genetic code, and which encode a nematode-inducible transmembrane pore protein.

34. A DNA construct according to claim 32, which promoter is constitutively active in plant cells.

35. A DNA construct according to claim 32, which promoter is selectively active in plant root tissue cells.

36. A DNA construct according to claim 32, which promoter is activated by a plant-parasitic nematode.

37. A DNA construct according to claim 32, which promoter is a nematode-responsive element selected from the group consisting of:

(i) isolated DNA having the sequence given herein as SEQ ID NO:5; and

(ii) isolated DNA which hybridizes to isolated DNA of (i) above and which encodes a nematode responsive element.

-43-

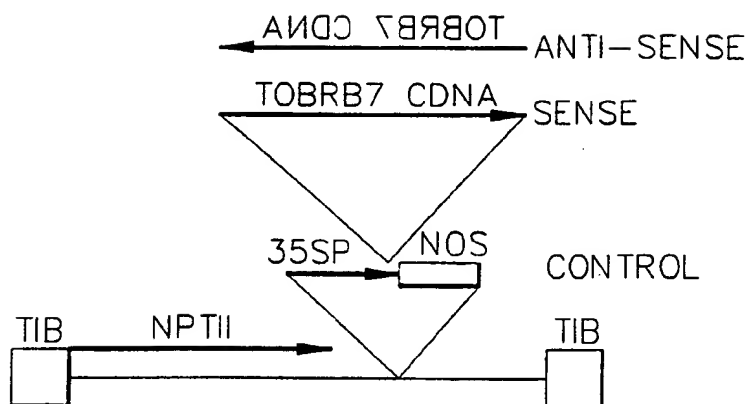
38. A nematode-resistant transgenic plant comprising plant cells containing a DNA construct according to claim 32.

39. A crop comprising a plurality of plants according to claim 38 planted together in an agricultural field.

40. A method of combatting a plant parasitic nematodes in an agricultural field, comprising planting the field with a crop of plants according to claim 38.

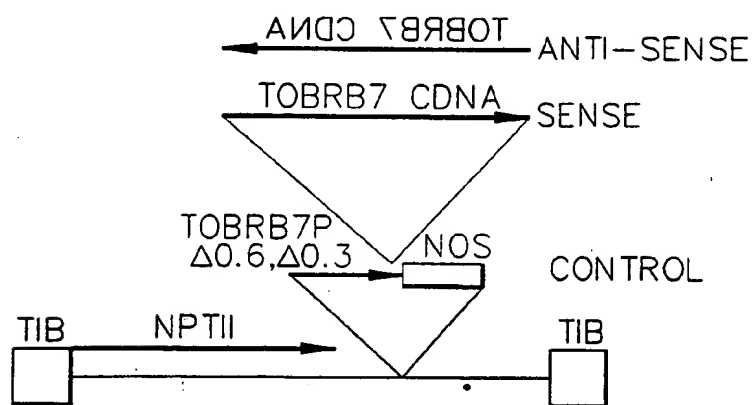
41. A method of making a recombinant pathogen-resistant plant, said method comprising:  
providing a plant cell capable of regeneration;  
transforming said plant cell with a DNA construct according to claim 32; and then  
regenerating a recombinant nematode-resistant plant from said transformed plant cell.

1/1



CONSTITUTIVE EXPRESSION OF SENSE AND ANTI-SENSE TOBRB7

FIG. 1.



TISSUE-SPECIFIC EXPRESSION OF SENSE AND ANTI-SENSE TOBRB7

FIG. 2.

# INTERNATIONAL SEARCH REPORT

Inter.    nal Application No  
PCT/US 94/00217

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 5    C12N15/82    A01H5/00    A01N65/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 5    C12N    C07K    A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THE PLANT CELL vol. 3 , 1991 pages 371 - 382 Y.T. YAMAMOTO ET AL.; 'Characterization of cis-acting sequences regulating root-specific gene expression in tobacco' *pages 372, 374 and 375*	1-3, 7-20, 22-26
A	WO,A,92 04493 (THE UNIVERSITY OF LEEDS) 19 March 1992 *claims*	1
A	WO,A,92 21757 (PLANT GENETIC SYSTEMS, N.V.) 10 December 1992 *claims*	1
	--- -/-- ---	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- '&' document member of the same patent family

Date of the actual completion of the international search

6 June 1994

Date of mailing of the international search report

24. 06. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+ 31-70) 340-3016

Authorized officer

Yeats, S



# INTERNATIONAL SEARCH REPORT

Inter nal Application No

PCT/US 94/00217

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO,A,93 06710 (NORTH CAROLINA STATE UNIVERSITY) 15 April 1993  *whole document* ---	1-3, 7-20, 22-31
P,X	WO,A,93 10251 (MOGEN INTERNATIONAL N.V.) 27 May 1993 *pages 9-30; example III, m) - r); claims* ---	1-31
P,X	SCIENCE vol. 263 , 1994 pages 221 - 223 C.H. OPPERMAN ET AL.; 'Root-knot nematode-directed expression of a plant root-specific gene' *whole document* -----	1-41

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/US 94/00217

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9204493	19-03-92	CN-A-	1060322	15-04-92
		EP-A-	0500862	02-09-92
		JP-T-	5502403	28-04-93
		PT-A-	98835	30-11-93
		US-A-	5259329	09-11-93
-----				
WO-A-9221757	10-12-92	CA-A-	2110169	10-12-92
		EP-A-	0586612	16-03-94
-----				
WO-A-9306710	15-04-93	AU-A-	2872692	03-05-93
		CA-A-	2112999	15-04-93
		PT-A-	100930	29-10-93
-----				
WO-A-9310251	27-05-93	AU-A-	2928492	15-06-93